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14. ABSTRACT

We sought to determine whether decreasing FMRP expression in Xenopus may help understand the consequences of loss of FMRP. We tested the effect of knocking down FMPR expression on visually guided behavior, seizure and brain development.
We established a highly sensitive quantitative in vivo imaging assay to evaluate molecular genetic strategies to decrease FMRP expression in brain neurons and demonstrated the capacity to rescue the decreased FMRP expression by gene delivery. We characterized an innate visually-guided avoidance behavior in tadpoles and showed that the avoidance behavior shows rapid and long-lasting improvement after brief periods of training. Decreasing FMRP expression does not significantly impair visual avoidance behavior. We developed a second behavioral assay to evaluate the loss of FMRP in which animals are exposed to seizure-inducing drugs. Decreased FMRP expression increases seizure latency, which was partially compensated by gene delivery of an FMRP homolog. We demonstrated that knocking down FMRP expression in neural progenitor cells decreases neurogenesis, and that knocking down FMRP expression in differentiating neurons blocks the development of neuronal dendritic arbors. Our experiments are the first to demonstrate that loss of FMRP causes deficits in neurogenesis during brain development and indicate that these events may be important to target for novel therapeutics. Our studies demonstrate that Xenopus is a valuable system in which to model Fragile X Syndrome.

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Introduction

Fragile X Syndrome is a neurodevelopmental disorder affecting 1 in 4000 males and 1 in 6000 females worldwide and is the leading cause of inherited intellectual disability. Fragile X Syndrome can also include autistic behavior, heightened sensitivity to sensory stimulation, and seizure. Fragile X Syndrome is caused by mutations in the Fragile X Mental Retardation 1 (FMR1) gene that prevents expression of its protein product, Fragile X Mental Retardation Protein (FMRP). A vertebrate experimental system in which loss of function of FMRP results in behavioral deficits has not been established. Such a system could be valuable to understand mechanisms contributing to deficits in Fragile X Syndrome. The Xenopus tadpole is a unique model system that allows easy access to the nervous system at early stages of development, is amenable to in vivo gene manipulation and gene therapies, and displays behavioral phenotypes that can be altered with genetic manipulations or bath application of drugs that can be absorbed directly through the animal's skin. We sought to establish Xenopus laevis as a model system to study consequences of loss of FMRP on brain function and behavior, and to use this system to identify candidate genes that might rescue behavioral deficits that arise from lack of FMRP. We established quantitative in vivo imaging methods to knockdown and assay synthesis of FMRP in Xenopus tadpole brains. We also established 2 behavioral assays to evaluate the effects of FMRP knockdown. One assay is a visually-guided avoidance behavior, which improves following behavioral training. The other is an assay of response to seizure-inducing drug, in which we quantify latency to start of seizure as well as other behavioral parameters. Finally, we established methods to evaluate a potential role for FMPR in neurogenesis and brain development. Progress described below demonstrates that this experimental system may provide insight into Fragile X Syndrome and treatment in people.

Task 1. Test whether knockdown of FMR1 alters tadpole behavior

1a. Validate knockdown of FMR1 by morpholino.

Xenopus express homologs of the Fragile X genes, *fmr1a* and *fxr1*, in their developing nervous system (Lim et al., 2005), allowing identification of basic neural mechanisms relevant to the human neurological disease, Fragile X Syndrome. To test whether knockdown of FMRP with the Fmr1a morpholino is efficient and specific, we developed a novel assay that provides a sensitive readout of translational knockdown in cells of interest in intact animals. We generated an expression construct that generates a single mRNA of *Xenopus* Fmr1b and eGFP separated by a T2A sequence. The T2A sequence, originally from the insect virus, Thosea asigna, induces ribosome skipping and initiation of a second polypeptide with ~100% efficiency when included in the mRNA transcript (Donnelly et al., 2001; Szymczak et al., 2004; Tang et al., 2009). The *fmr1b-t2a-egfp* transcript was expressed under the control of a minimum FGF promoter that includes a Sox2/Oct4 binding domain (bd). Transcription requires binding of endogenous Sox2/Oct4 transcription factors, and therefore results in protein expression in Sox2/Oct4 expressing neural progenitor cells, as described (Bestman et al., 2012). The expression plasmid includes gal4/UAS to amplify Fmr1-T2A-eGFP expression, and is designated as pSox2bd::gal4UAS Fmr1-T2A-eGFP. The plasmid generates a *gal 4* transcript and a separate transcript *UAS Fmr1-T2A-eGFP.* The gal4 protein binds the UAS sequence and initiates translation. We co-electroporated anesthetized stage 47 tadpoles with 2 expression plasmids, pSox2bd::gal4UAS Fmr1-T2A-eGFP and UAS::turboRFP$_{nls}$ (tRFP$_{nls}$). The tRFP is targeted to the nucleus by the nuclear localization signal (nls) and serves as in internal reference for protein expression driven from the pSox2bd::gal4. We also electroporated an antisense oligonucleotide morpholino against fmr1a (Fmr1MO) or a control morpholino (ContMO) at a concentration of 0.05mM. The Fmr1a morpholino will block translation of the Fmr1-t2A-eGFP transcript and will therefore result in decreased expression of both Fmr1 and eGFP in cells with knockdown without affecting expression of tRFP (Figure 1A). We identified the RFP-expressing cells and determined the proportion of RPF-expressing cells that had no detectable GFP. Animals electroporated with FmrMO had a significant increase in cells expressing RFP$_{nls}$ without detectable eGFP (Figure 1B, D) (RFP+/GFP- cells relative to total RFP+ cells: Fmr1MO 50% ± 8% n=23 tadpoles; ContMO 17% ± 4%, n=20 tadpoles; p<0.05).

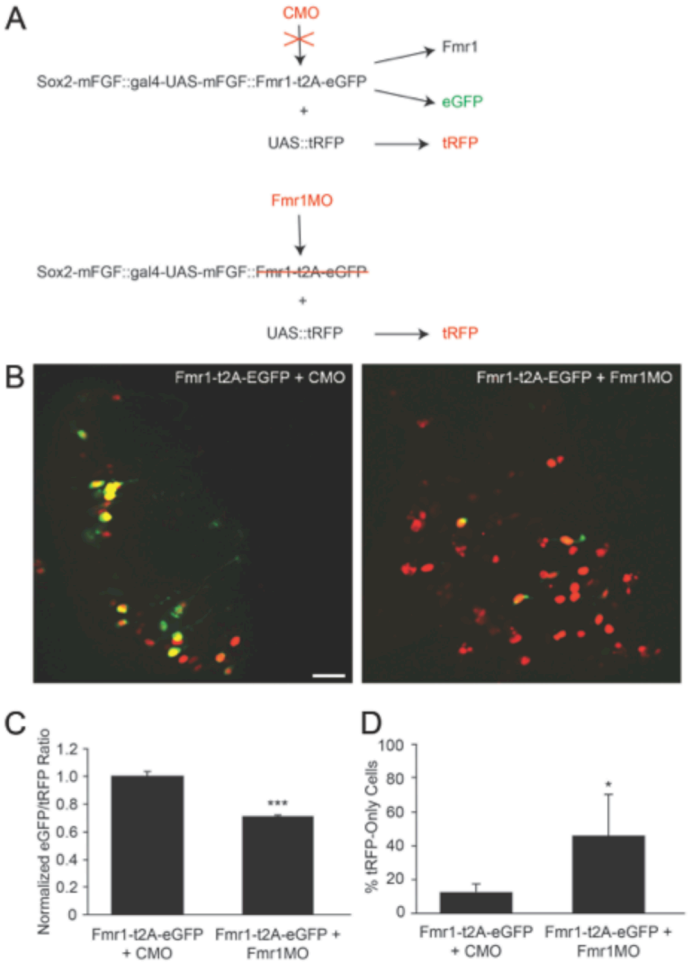


Figure 1. FMR1 morpholino decreases Fmr1 expression. (A) Schematic of the experimental paradigm. Co-electroporation of ContMO with the Fmr1-t2A-eGFP and UAS::tRFP constructs will result in expression of Fmr1, eGFP, and tRFP. In contrast, co-electroporation of Fmr1-T2A-eGFP and UAS::tRFP constructs with Fmr1MO should block translation of the Fmr1-T2a-GFP transcript, thereby reducing eGFP expression without affecting tRFP expression. Strong Fmr1-T2a-GFP knockdown will result in detection of RFP only. (B) Sample images of eGFP and tRFP expression with and without co-electroporation of Fmr1MO. (C) Quantification of the eGFP/tRFP ratio normalized to ContMO, shows a decrease in eGFP expression with Fmr1MO, demonstrating that Fmr1MO blocks Fmr1translation. (D) Quantification of the percentage of tRFP-expressing cells that lack detectable eGFP expression in animals electroporated with Fmr1MO. Cells that only express RFP have such strong knockdown that no GFP is detected. Scale bar = 29um. ***p<0.0001, *p<0.05.

Since ~50% of RFP+ cells did have detectable GFP expression we tested whether GFP expression was reduced in animals electroporated with FmrMO, since GFP serves as a proxy for Fmr1 expression because the 2 proteins are synthesized at equimolar quantities. We measured the fluorescence intensities of eGFP and tRFP in each cell, and normalized the ratio of eGFP/tRFP intensities to the average eGFP/tRFP per cell in ContMO animals. In animals electroporated with Fmr1MO, cells had significantly lower eGFP/tRFP ratios than cells from animals electroporated with ContMO (Figure 1B-C) (eGFP/tRFP ratio in ContMO: 1.00 ± 0.03, n=391 cells; Fmr1MO 0.71 ± 0.01, n=453 cells; p<0.0001). Together, these results demonstrate that Fmr1MO blocks translation of the Fmr1-t2A-eGFP transcript, resulting in fewer cells with eGFP expression (and by proxy, Fmr1 expression). In cells that express detectable levels of eGFP, those expression levels are decreased relative to RFP expression. We prepared a construct for rescue of Fmr1 expression that contains *Xenopus* Fmr1b with silent mutations rendering it insensitive to the morpholino (ΔxFmr1). When ΔxFmr1-t2A-eGFP was co-electroporated with Fmr1MO, we detected no decrease in the eGFP/tRFP ratio compared to ContMO, confirming that it is insensitive to the morpholino and can be used to rescue knockdown of Fmr1 in our experiments (ΔxFmr1 + CMO 1.00 ± 0.03, n=335 cells; ΔxFmr1 + Fmr1MO 1.08 ± 0.03, n=304 cells). The data are presented as average ± SEM and a Student's T-test was used to determine significance.

1b. Test visually-guided avoidance behavior.

Tadpoles escape from an approaching object. This innate tectally-mediated visually-guided avoidance behavior is assessed as a change in swim trajectory when a moving spot enters the animal's visual field at approximately right angles to the eye (Figure 2A). We used the avoidance index (% of avoidance responses per 10 trials) to quantify the avoidance success rate when tadpoles encounter moving spots (Dong et al., 2009; McKeown et al., 2013; Shen et al., 2011). We assessed visual avoidance over 4-24 hours by measuring avoidance during 1-minute test periods with half an hour intervals between tests. We did not observe any habituation of the avoidance index when Xenopus was tested for avoidance over 7 hours (Figure 2B), indicating our assay is suitable for studies of behavioral plasticity over this time frame.

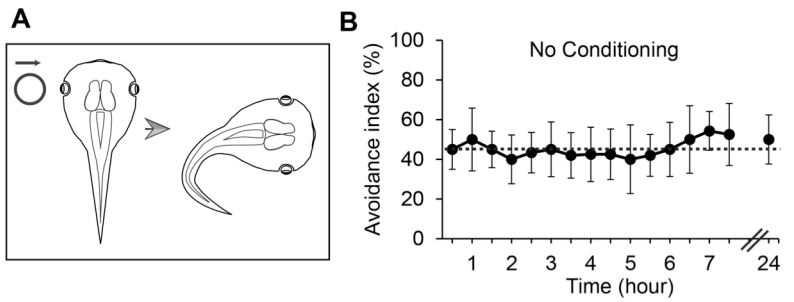


Figure 2. Visual avoidance behavior (A) An illustration of the visual avoidance behavior in response to a moving spot approaching the eye. The tadpole is swimming forward when it encounters a spot moving toward it from left to right (small arrow represents the direction of movement of the spot). In response to the stimulus, the animal changes it swim trajectory by turning sharply to the left or right, called avoidance behavior. (B) The avoidance index in response to 0.4 cm moving spots remains constant over 7 hours when animals are tested for 1 minute every 0.5 hour. The avoidance index remains unchanged after 24 hours. Dotted line is the average of the avoidance index of first three tests. N=8 animals.

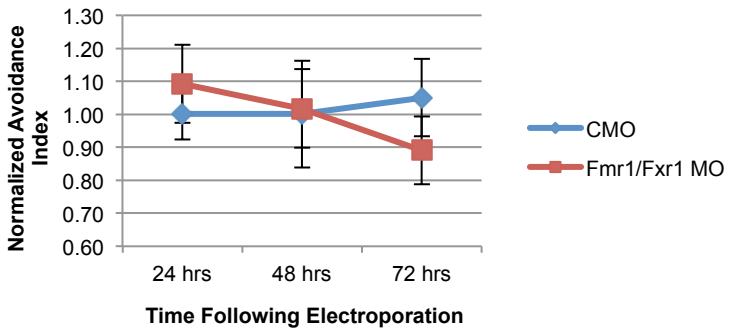


Figure 3. Knockdown of Fmr1a and Fxr1 does not affect tadpole avoidance behavior for up to three days following electroporation of Fmr1a and Fxr1 antisense morpholinos.

To test whether Fmr knockdown can affect an innate behavior, we tested visually-guided avoidance behavior in stage 47 Xenopus tadpoles after they were co-electroporated with morpholinos against Fmr1a and Fxr1. Fxr1 is an autosomal paralog of Fmr1a that is highly similar and might be functionally redundant with Fmr1a. To eliminate this possible redundancy, we knocked down both Fmr1a and Fxr1. We

screened stage 47 animals for the optomotor response to assess normal swimming behavior. Animals passing the optomotor screen were electroporated with antisense morpholinos against Fmr1a and Fxr1 (Fmr1/Fxr1MO), or control morpholino (ContMO), at a stock concentration of 0.05mM. One, two and three days later, tadpoles were placed in a clear tank and randomly moving dots were presented for 90 sec using a microprojector positioned below the tank, as described (Shen et al., 2011). Videos of tadpole movements were recorded and analyzed for encounters with dots approaching the eye perpendicularly. Only animals having at least 5 encounters during the 90 sec exposure period were included in the analysis. The percent of encounters that gave a turning response within 500ms of the encounter was called the Avoidance Index. To control for clutch to clutch variation in animal behavior, Avoidance Indices for each group and time point were normalized to the average Avoidance Index of the matched control group taken one day after electroporation for each experiment. We found no significant effect of Fmr1a and Fxr1 knockdown on the Avoidance Index at the time points tested (Figure 3) (24 hrs: ContMO 1.00 ± 0.08 vs Fmr1/Fxr1MO 1.09 ± 0.12; 48 hrs: ContMO 1.00 ± 0.16 vs Fmr1/Fxr1MO 1.02 ± 0.12; 72 hrs: ContMO 1.05 ± 0.12 vs Fmr1/Fxr1MO 0.89 ± 0.10). The data are presented as average ± SEM and a Student's T-test was used to determine significance.

1c. Test improvement of visually-guided avoidance behavior with training.

We tested the effects of several different protocols for visual conditioning on the innate tectally-mediated visual avoidance behavior (Figure 4). We exposed freely swimming animals to a stimulus composed of bars moving at 0.3 Hz in 4 directions in pseudorandom order and tested the visual avoidance index in response to moving spots of 0.6, 0.4, 0.2, and 0.1 cm in diameter. Previous experiments showed that the visual avoidance response is maximal for 0.4 cm moving spots (Dong et al., 2009; Shen et al., 2011). Exposure to 30 minutes of conditioning consisting of 3 five-minute episodes of moving bars with 5-minute intervals without stimulus between episodes resulted in long-lasting enhancement of the behavioral response (Figure 1C). The avoidance index was measured three times at 30-minute intervals to establish a baseline avoidance index before tadpoles were exposed to the visual conditioning. The avoidance index was determined every 30 minutes over the next 4 hours to evaluate the effect of conditioning. A significant increase in the avoidance index was detected 1.5 hour after 30 minute of visual conditioning. The improvement of the avoidance index was maintained for 24 hours (Figure 1C). Exposure to for 2 or 4 hours of continuous visual conditioning (VC) significantly improved the avoidance response when tested 30 minutes or 1 day after the end of the conditioning period (Figure 1D, E). Visual conditioning did not significantly affect responses to other spot sizes (Supplementary Figure 1A, 1B). These results indicate that the visual avoidance response is plastic in response to brief exposure to visual conditioning, that the plasticity can be detected shortly after conditioning and is maintained for at least one day. We used this conditioning protocol in the following experiments investigating the mechanisms underlying visual avoidance plasticity.

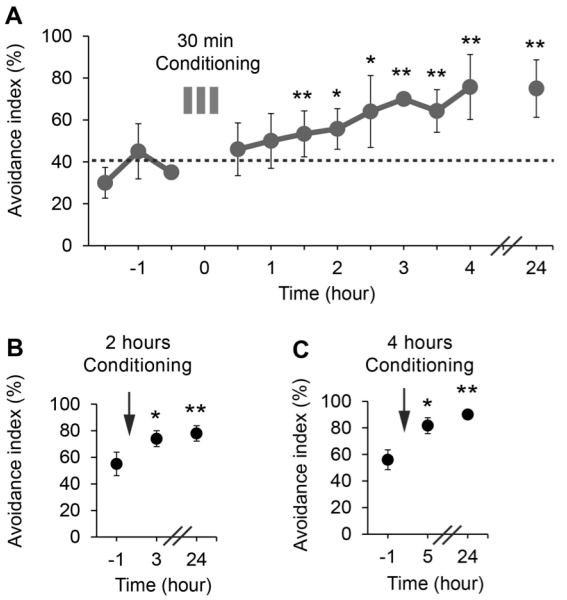


Figure 4 Experience-dependent improvement of visual avoidance behavior. A. Conditioning for 30 minutes (5 minutes on, 5 minutes off for 3 episodes) during the period marked with the grey bars significantly enhanced avoidance index in response to 0.4 cm moving spots. The improvement of avoidance index was maintained until 24 hours after conditioning (N=8, **P<0.01, *P<0.05). B,C. The avoidance index is significantly increased when tested 30 minutes (N=6, *P<0.01, green line) or 24 hours (N=6, **P<0.05, red line) after 2 hours (B) or 4 hours (C) conditioning, compared to control tadpoles before conditioning.

We tested whether knockdown of Fmr1a will affect the ability of animals to improve performance in the avoidance assay following short term visual enhancement (STVE) provided by exposure to a simulated motion stimulus comprised of rows of LEDs that are sequentially turned on and off. Stage 47 animals were screened for the optomotor response. Animals passing the optomotor screen were electroporated with an antisense morpholino against Fmr1a (Fmr1MO), or control morpholino (CMO), at a concentration of 0.05mM. 3 days following electroporation, animals were presented with moving dot stimuli for 90s as described above. Then, animals were presented with STVE for 4 hours. Following STVE, animals were presented once again with the moving dot stimuli for 90s. This moving dot-STVE-moving dot paradigm was repeated again 24 hours later. Videos of tadpole movements were recorded and the Avoidance Index was quantified as described above. To control for clutch to clutch variation, Avoidance Indices were normalized to the average Avoidance Index of the ContMO group before STVE for each experiment. We found no significant effect of Fmr1 knockdown on STVE-induced improvement in the Avoidance Index (Figure 5) (Pre STVE Day 1: CMO 1.00 ± 0.19 n=12, Fmr1MO 1.04 ± 0.27 n=12; Post STVE Day 1: CMO 1.21±0.31 n=12, Fmr1MO 1.18 ± 0.33 n=6; Pre STVE Day 2: CMO 1.16 ± 0.2 n=11, Fmr1MO 1.39 ± 0.21 n=10; Post STVE Day 2: CMO 1.47 ± 0.23 n=11, Fmr1MO 1.61 ± 0.15 n=6). The data are presented as average ± SEM and a Student's T-test was used to determine significance.

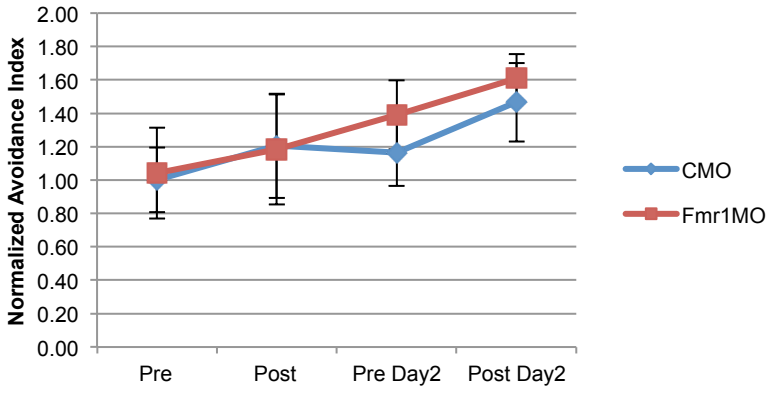


Figure 5. Knockdown of Fmr1a does not affect improvement in visual avoidance behavior seen with visual conditioning.

1d. Test susceptibility to drug-induced seizure.

We tested whether knockdown of Fmr1a alters susceptibility to PTZ-induced seizure. Stage 41-43 animals were electroporated with antisense morpholino against Fmr1a (Fmr1MO) or control morpholino (CMO). Three days later, animals were exposed to 15mM PTZ in rearing solution for 20 min. Videos of tadpole movements were recorded every 2 min for 30 sec and analyzed post hoc. The latency between drug exposure and onset of seizure, defined as a C-shaped body contraction, was quantified. To control for clutch to clutch variation, seizure latencies were normalized to the average control seizure latency for each experiment. We found that Fmr1 knockdown significantly increased seizure latency compared to CMO (Figure 6) (CMO 1.00 ± 1.07 n=57, Fmr1MO 1.39 ± 0.10 n=56, p=.01). The data are presented as average ± SEM and statistical significance was determined by ANOVA followed by a Tukey-Kramer test.

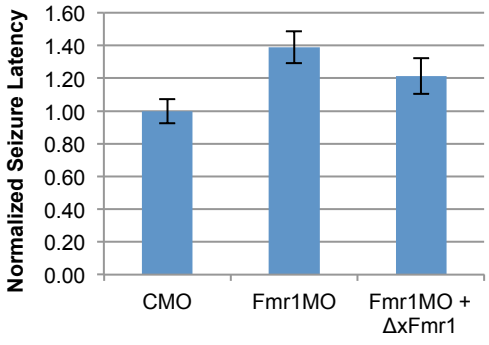


Figure 6. Knockdown of Fmr1a increases drug-induced seizure latency compared to controls. This effect on seizure latency can be partially rescued by introducing a morpholino-insensitive Fmr1 construct (ΔxFmr1).

1e. Test rescue of FMR1A knockdown with FMRP.

We found that co-electroporation of Fmr1MO with a construct containing *Xenopus* Fmr1b with a silent mutation rendering it insensitive to morpholino (ΔxFmr1), could partially rescue the seizure latency defect caused by

Fmr1a morpholino (Figure 6) (Fmr1MO + ΔxFmr1 1.21 ± 0.11 n=45). The data are presented as average ± SEM and statistical significance was determined by ANOVA followed by a Tukey-Kramer test.

Task 2. Test rescue of FMR1A knockdown with 4 candidate genes: CPEB, pumilio, staufen, Fxr1.

2a. Test visually-guided avoidance behavior and improvement with training.

We found no defects in visual avoidance behavior or training-induced improvement in visual avoidance with knockdown of Fmr1a (Figure 5).

2b. Test susceptibility to drug-induced seizure.

To test the ability of other RNA binding proteins to rescue the Fmr1a knockdown-mediated defect in seizure latency, we co-electroporated Fmr1MO with expression constructs for either *Xenopus* Fxr1 or *Xenopus* CPEB. Stage 42-43 animals were electroporated with antisense morpholino against Fmr1a (Fmr1MO) or control morpholino (CMO) at a concentration of 0.05mM. A subset of animals electroporated with Fmr1MO were also electroporated with expression constructs for Fxr1 or CPEB. Three days later, animals were exposed to 15mM PTZ in rearing solution for 20 min. Videos of tadpole movements were recorded and analyzed for seizure latency as described above. As shown above, Fmr1 knockdown significantly increased seizure latency compared to CMO (CMO 1.00 ± 0.2 n=7, Fmr1MO 2.07 ± 0.32 n=8, $p<0.05$). Furthermore, co-electroporation of Fmr1MO with Fxr1 partially rescued Fmr1 knockdown (Fmr1MO + Fxr1 1.60 ± 0.22 n=6, $p=0.437$ compared to CMO), while co-electroporation of Fmr1MO with CPEB had no effect on seizure latency (Fmr1MO + CPEB 2.26 ± 0.29 n=6, $p<0.05$ compared to CMO) (Figure 7). The data are presented as average ± SEM and statistical significance was determined by ANOVA followed by a Tukey-Kramer test. Our previous work has shown that overexpression of CPEB can be as detrimental as CPEB knockdown for neuronal development, establishment of circuit connectivity and visual information processing (Bestman and Cline, 2008). Since FMR is a negative regulator of protein translation, loss of FMRP increases protein translation. Once activated, endogenous levels of CPEB enhance translation and, because CPEB and FMRP have many of the same mRNA cargo, CPEB-mediated translation may result in further overexpression of some proteins in FMRP knockdown conditions. It is interesting to note that a recent study reported that CPEB knockdown rescued behavioral deficits in a mouse model of Fragile X (Udagawa et al., 2013). Recent bioinformatic studies suggest that mRNA cargo of Pumilio and Staufen are not highly overlapping with FMRP mRNA cargo, making it unlikely that manipulating their expression will rescue loss of FMRP.

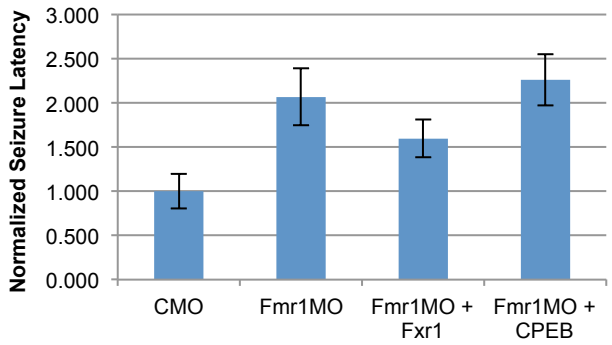


Figure 7. The Fmr1MO-mediated defect in seizure latency can be partially rescued by co-expression of Fxr1, but not CPEB.

Task 3. Test rescue of FMR1A knockdown with a subset of drugs from the Spectrum Collection.

Since we found no defects in visual avoidance behavior or training-induced improvement in visual avoidance with knockdown of Fmr1a we did not test the drugs from the Spectrum Collection. Instead we developed a sensitive assay of in vivo neurogenesis and neuronal integration into the developing visual circuit, in an effort to establish an alternate way to investigate the effect of FMRP loss on brain development.

The majority of research into the deficits arising from loss of FMRP has focused on the hypothesis that Fragile X Syndrome arise from deficits in synaptic plasticity, yet recent studies indicate that neurogenesis, the generation of neurons from progenitor cells, is abnormal in patients with FXS (Parrini et al., 2006). Brain development requires strict spatial and temporal regulation of cell proliferation, differentiation, survival and migration, so errors in the regulation of neurogenesis will have profound effects on brain development and function.

We investigated the function of FMRP and FXR1P in neural cell proliferation and differentiation in the central nervous system of intact *Xenopus laevis* tadpoles. We hypothesized that loss of FMRP or FXR1P in the tadpole nervous system will decrease the rate of proliferation and decrease neuronal differentiation.

Notably, *Xenopus laevis* tadpoles are transparent and develop externally, enabling direct observation of neurogenesis in early developmental stages (Bestman et al., 2012). Stage 46 animals were anesthetized and electroporated with Sox2mFGF4::GFP to drive GFP expression in neural progenitor cells. Either a control morpholino that does not affect protein expression or antisense morpholinos, which knockdown expression of FMR1 or FXR1, were also electroporated. The following day, animals were anesthetized and imaged using a spinning disk confocal microscope to produce 3 dimensional images of the tadpole brain in which neural progenitor cells are GFP-labeled (Figure 7). The animals were imaged every 24 hours over a 3-day period. The images were analyzed by counting the total number of labeled cells per brain hemisphere for each tadpole. Counted cells are then identified as either mature neurons or radial glia progenitor cells based on established morphological features.

Animals treated with control morpholinos increase in the number of GFP-labeled cells between the first and third days of imaging, but FMR1 or FXR1 knockdown significantly decreased the total number of GFP-labeled cells generated over the imaging period. After identifying neurons and glia based on their morphology we found that the average number of neurons and radial glia progenitor cells on days 2 and 3 is significantly reduced with FMR1 or FXR1 knockdown compared to controls, suggesting that proliferation and survival of neural progenitor cells is compromised by loss of Fragile X proteins (Figure 8). These data indicate that loss of FMRP (1) decreases neural progenitor cell survival, and/or (2) reduces progenitor proliferation and (3) induces rapid differentiation of neurons from progenitors.

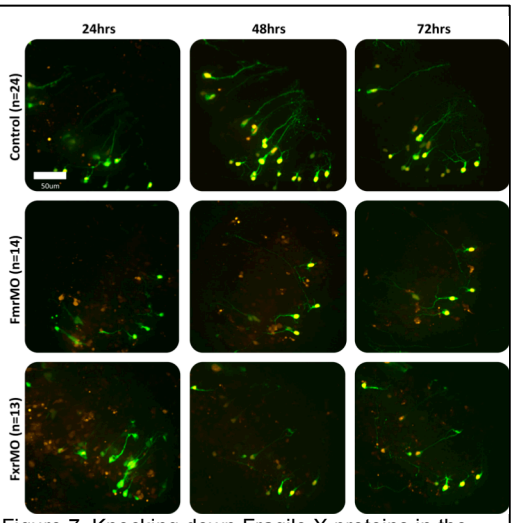


Figure 7. Knocking down Fragile X proteins in the developing brain interferes with neurogenesis. In vivo time-lapse images of GFP-expressing neural progenitors and their neuronal progeny collected over 2 days in the brains of intact anesthetized tadpoles.

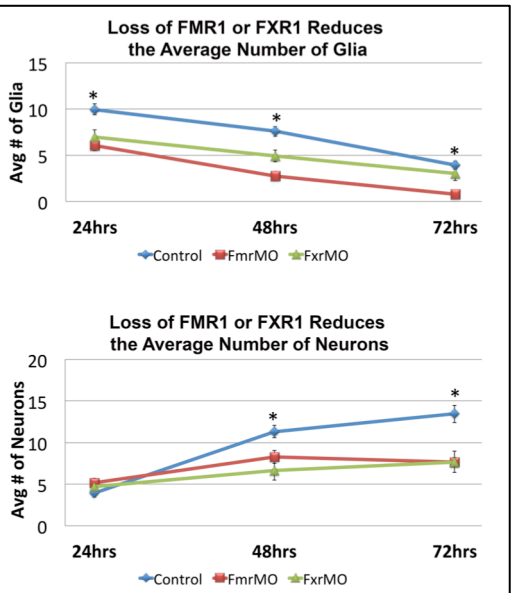
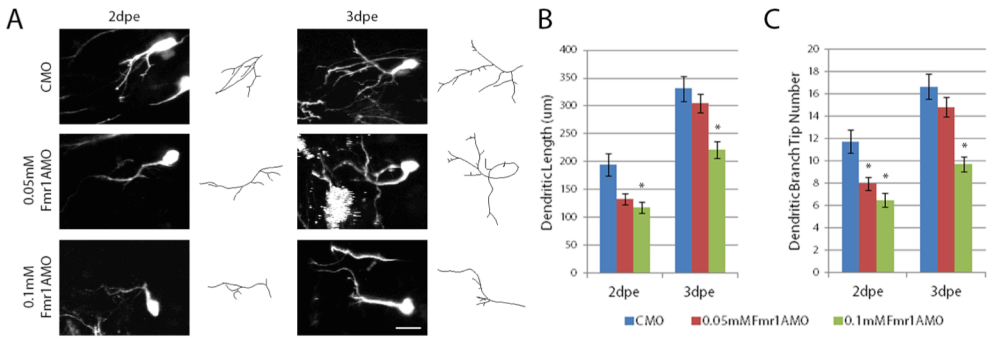


Figure 8. Knocking down Fragile X proteins in the developing brain decreases neurogenesis by 2 distinct mechanisms. Top. Loss of FMR1 or FXR1 reduces the number of neural progenitor cells in the brain. Bottom. Loss of FMR1 or FXR1 reduces the number of differentiated neurons.

We noticed that the dendritic arbors of neurons in animals with FMRP knockdown appeared malformed and smaller than neurons in control animals. We therefore analyzed the dendrite morphologies quantitatively and found that loss of FMRP severely impairs dendrite development (Figure 9). Although classic work in the field showed that excitatory spine synapses are aberrant in pyramidal neurons in people with Fragile X Syndrome and animals models of Fragile X Syndrome, relatively little work has been done investigating the potential function of FMRP on early stages of neuronal differentiation. Our data indicate the FMRP is required for neurogenesis and for neuronal development. The data add insight into the mechanisms by which Fragile X Syndrome affects brain function and suggest promising directions for future research.



Fmr1A regulates dendritic development. A. Time-lapse images were collected 2dpe and 3dpe on a custom-built 2-Photon microscope using a 20X objective lens (scale bar = 20um). The images that are shown are not time-lapse, but are representative of the CMO, 0.05mM Fmr1AMO, and 0.1mM Fmr1AMO groups at each time point. Dendritic arbors were reconstructed (right) and measurements of dendrite length (B) and branch tip number (C) were recorded using Imaris. (B,C) Knockdown of Fmr1A with 0.05mM MO resulted in a decrease in dendritic branch tip number at 2pde, but this decrease was no longer apparent at 3dpe. Knockdown of Fmr1A with 0.1mM MO resulted in decreases in both dendritic length and branch tip number at 2dpe that was maintained at 3dpe. * $p < 0.05$. For statistical analysis we used ANOVA followed by a Tukey's HSD test to determine significance.

Key Research Accomplishments

- Establish a quantitative in vivo imaging assay to evaluate protein knockdown in neurons
- Develop and test reagents to manipulate FMRP protein expression in vivo
- Document decreased FMRP synthesis and rescue of loss of protein by gene therapy
- Establish quantitative behavioral assays to evaluate consequences of decreased FMRP expression or rescue by gene delivery methods
- Demonstrate improvement in visual avoidance behavior in response to training
- Demonstrate changes in behaviors in response to decreased FMRP synthesis and rescue by gene delivery
- Establish quantitative in vivo imaging assays to evaluate consequences of decreased FMRP expression or rescue on brain development
- Demonstrate effects of FMRP knockdown and rescue on brain development

Reportable Outcomes

- Established Xenopus as an experimental system to study Fragile X Syndrome with support from the DoD
- Developed new quantitative methods to evaluate gene knockdown in vivo
- Demonstrated that knockdown of Fragile X proteins affects several behaviors in Xenopus tadpoles
- Demonstrated that knockdown of Fragile X proteins affects neurogenesis in vivo and brain circuit development
- 2 manuscripts are in preparation including work accomplished with this DoD support
- The work has been/will be presented in abstract and poster presentations at 4 meetings: The 2014 ERN Conference in STEM, the 2014 SACNAS, the Fragile X Syndrome Gordon Conference in June 2014, and the Society for Neuroscience Annual Conference in November 2014.*
- Supported training of an undergraduate and a postdoctoral fellow
- Trainee obtained a MARC (Minority Access to Research Careers) fellowship based on research on this project
- Obtained funding from NIH partially based on preliminary data generated with the support of the DoD
- Launched a new collaborative project on FMRP effects on behavior

Conclusions

This study has resulted in significant progress toward our goal of establishing Xenopus as a model system to study deficits in brain development and function in Fragile X Syndrome. The most promising elements of the study are indications that decreasing FMRP expression interferes with learning visual processing behavior. We have initiated new experiments to evaluate a broader range of visually-guided behaviors in Xenopus under conditions of decreased FMRP expression. Recent work has shown that in human fetuses, Fragile X protein is initially expressed in neural progenitors and neurons, and then its expression is silenced. To mimic this pattern of expression followed by knockdown, we have tested the effect of knocking down FMRP expression in neural progenitors and neurons. We find that FMRP knockdown during CNS development interferes with neurogenesis and the development of visual information processing centers in the brain. These experiments show that Xenopus is a valuable experimental system to examine the etiology of Fragile X Syndrome, by mimicking the transient expression pattern of FMRP in human fetal brain. Our studies indicate that a promising route to investigate FMRP function is to probe its role in neurogenesis, neuronal differentiation and circuit assembly. The experiments funded by the DoD have provided a foundation for further investigation of novel mechanisms of brain development affected by loss of FMRP.

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Appendices

List of personnel receiving pay from the research effort

Hollis Cline, Ph.D. – Principle Investigator
Evan Fitchett B.S. – Research Technician
Jenifer Krass B.S. – Research Technician

The following abstract was submitted for:

Gordon Research Seminar and Conference: Fragile X and Autism–Related Disorders, May 31-June 6, 2014

Dysregulation of Fragile X Mental Retardation Protein during early embryonic development results in defective neurogenesis in *Xenopus laevis* tadpoles.

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ABSTRACT:
Fragile X Syndrome (FXS) is the leading known monogenic form of Autism and the most common form of inherited intellectual disability. Developmentally-regulated loss of function of the *fmr1* gene results in lack of Fragile X Mental Retardation Protein (FMRP), an RNA binding protein. Recent studies suggest that neurogenesis, the generation of neurons from progenitor cells, is aberrant in FXS patients. We investigated whether FMRP affects neurogenesis, using *Xenopus laevis* tadpoles which express a homolog of the *fmr1* gene. We knocked down FMR1 using antisense morpholinos and collected *in vivo* confocal time-lapse images of GFP-expressing radial glial progenitor cells and their neuronal progeny over three days. Animals treated with control morpholinos have an increase in the number of GFP-labeled cells between the first and third days of imaging, but FMR1 knockdown decreased the total number of GFP-labeled cells generated over the imaging period, suggesting that proliferation and survival of progenitor cells is compromised by loss of FMRP. Furthermore, we identified neurons and radial glial cells based on their morphology, and found the average number of neurons was significantly reduced on the third day of imaging, whereas the average number of radial glial cells was significantly reduced across all three days of imaging, with FMR1 knockdown compared to controls. Together these data suggest that loss of FMRP reduces differentiation of neurons from progenitors. Lastly, to test whether the effects of FMR1 knockdown were specific to the *fmr1* gene, we simultaneously knocked down FMR1 and overexpressed the gene using a morpholino-insensitive expression construct and found the defects in neurogenesis were restored to control levels. These data demonstrate that FMRP plays an important role in proliferation and differentiation, giving new insight into the pathophysiology of FXS.

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and

Emerging Researchers National Conference: NSF, US Dep't of Education & AAAS, February 20-22, 2014

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Fragile X Syndrome (FXS) is the most common form of inherited intellectual disability. Loss of function of the fmr1 gene results in lack of Fragile X Mental Retardation Protein (FMRP), an RNA binding protein. Two homologs of FMR1, FXR1 and FXR2 are expressed in brain and m ay have functional redundancy in RNA binding, but little is known about their role in development. Recent studies suggest that neurogenesis, the generation of neurons from progenitor cells, is aberrant in FXS patients. We investigated whether Fragile X proteins affect neurogenesis, using Xenopus laevis tadpoles which express homologs of fmr1 and fxr1 genes. We knocked down FMR1 and FXR1 with antisense morpholinos and collected in vivo confocal time lapse images of GFP-expressing radial glial progenitor cells and their progeny over three days. Animals treated with control morpholinos increase in the number of GFP-labeled cells between the first and third days of imaging, but FMR1 or FXR1P knockdown significantly decreased the total number of GFP-labeled cells generated over the im aging period. We identified neurons and glia based on their morphology and found that the average number of neurons and radial glia cells on days 2 and 3 is significantly reduced with FMR1 or FXR1 knockdown com pared to controls, suggesting that proliferation and survival of neural progenitor cells is com promised by loss of Fragile X proteins. Interestingly, knockdown of FMRP increased the proportion of neural progeny com pared to progenitors, suggesting that loss of FMRP induces rapid differentiation of neurons from progenitors, adding insight into mechanisms of FXS.

Supported by TJW (5T34GM087193)

This abstract was submitted for the SFN 2014 annual conference.

Control/Tracking Number: 2014-S-9005-SfN
Activity: Scientific Abstract
Current Date/Time: 5/8/2014 12:42:46 PM

Visualization of the newly-synthesized proteins required for synaptic plasticity in Xenopus laevis

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Abstract:
Synaptic plasticity, the cellular basis of learning and memory, is dynamic at both transcriptional and translational levels. We are interested in how protein synthesis is regulated with changes in synaptic transmission in response to visual stimuli or disease conditions. Unbiased investigation of global protein synthesis is challenging due to the lack of available techniques. We adapted a new technique, fluorescent non-canonical amino acid tagging (FUNCAT), a nonbiased labeling for newly-synthesized proteins, to examine the distribution of newly-synthesized proteins in Xenopus laevis. The localization of newly-synthesized proteins was ubiquitous in the tadpole brains but the fluorescence intensity varied between different cell populations and the neuropil. The neural progenitors, labeled by SOX2, have higher intensity labeling, indicating that the amount of translation may vary between cell-types in the developing brain. In addition, changes in protein synthesis were detected when animals were exposed to anisomycin, a protein translation inhibitor, and pentylenetetrazol (PTZ), a GABA receptor antagonist, known to elevate brain activity and to induce seizure. Currently, we are using FUNCAT to examine the changes of global protein synthesis in animals, which (1) are exposed to visual stimuli that are known to induce synaptic plasticity or (2) are electroporated with morpholinos to knock down proteins known to regulate protein translation and synaptic plasticity.
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Additional Conflict?: No

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